SUPPLEMENT

Beryllium-Ferritin: Lymphocyte Proliferation and Macrophage Apoptosis in Chronic Beryllium Disease

by

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Materials and Methods

Study Population: Written, informed consent was obtained from each patient enrolled in this study and the protocol was approved by the Human Subject Institutional Review Board at National Jewish Medical and Research Center, Denver, CO. CBD (n = 8) and beryllium sensitized (BeS) (n = 19) patients were consecutively enrolled based on the availability of BAL samples. The diagnosis of CBD was established using previously defined criteria, including of a history of Be exposure, the presence of granulomatous inflammation and/or mononuclear cell infiltration on lung biopsy, and a positive proliferative response of blood and/or BAL T cells to $BeSO_4$ stimulation *in vitro* [E1-E3]. The diagnosis of BeS was established based on a history of Be-exposure, a repeated positive proliferative response of blood T cells to $BeSO_4$ stimulation *in vitro*, and the absence of granulomatous inflammation on lung biopsy [E1-E3].

Chemicals and Reagents: Carrier-free $^{7}BeCl_2$, specific activity 2.6 mCi/mg at 1.66 mCi/ml, was purchased from Oak Ridge National Laboratory (Oak Ridge, TN) and counts per minute determined using a Packard Cobra Auto-gamma counter (Downers Grove, IL). Ferritin was purchased from Sigma Chemical Co. (St. Louis, MO). Beryllium

sulfate (Brush Wellman, Inc., Cleveland, OH) and aluminum sulfate (Sigma) were maintained at 4°C as stock solutions of 1 X 10^{-3} M $BeSO_4$ or 1 X 10^{-3} M $Al_2(SO_4)_3$ in water and diluted 1:10 or 1:100 during cell culture for final concentrations of 100 μM and 10 μM, respectively. Phytohemagglutinin (PHA, Sigma) was used as a positive control for T cell proliferation in the clinical Be lymphocyte proliferation test (BeLPT) [E1]. Phycoerythrin-labeled anti-CD95, FITC anti-CD71, FITC anti-CD4 and their corresponding labeled isotype control antibodies were purchased from BD-Biosciences (San Diego, CA).

Four test reagents were prepared including: 1) A beryllium-ferritin adduct (Be-ferritin) reagent prepared, as described by Price and Joshi [E4], using 1 mg/ml of ferritin and 0.1 M $BeSO_4$. 2) A ferritin protein control reagent prepared using 1 mg/ml of ferritin, but without $BeSO_4$. 3) A dialysis control reagent prepared using 0.1 M $BeSO_4$, but without the ferritin. 4) A ^{7}Be-ferritin adduct reagent prepared using radio-$^7BeCl_2$ and 1 mg/ml of ferritin, as above [E4].

The beryllium-ferritin adduct reagent was prepared by suspending one mg of ferritin in 1 ml of 0.2 M Tris-acetate, pH 6.5, plus 0.1 M $BeSO_4$ [E4]. The ferritin protein control reagent was prepared by suspending one mg of ferritin alone in 1 ml of 0.2 M Tris-acetate, pH 6.5. The dialysis control reagent was prepared using one ml of 0.2 M Tris-acetate plus 0.1 M $BeSO_4$ in the absence of ferritin. To determine the amount of Be bound by ferritin, the fourth ^{7}Be-ferritin adduct reagent was prepared by suspending 1 mg of ferritin in 1 ml of 0.2 M Tris-acetate, pH = 6.5, plus 0.2 mCi of $^7BeCl_2$.

The ferritin protein control reagent was used to insure that equal amounts of this iron-transport and storage protein were not toxic for the test cells in culture. The dialysis

control reagent was used to insure the efficiency of the Be-ferritin dialysis step (below), by removing the added 0.1 M $BeSO_4$ from the dialysis tubing at least to amounts of Be that were less than the amount of Be present in the Be-ferritin adduct. The Be-ferritin adduct, ferritin protein control, dialysis control and ^{7}Be-ferritin adduct reagents were then incubated for 15 min at 37^{o}C and the reagents transferred to individual, pre-prepared dialysis tubing (MW cut of = 5,000 Da). Each reagent was dialyzed in separate 2 X 1L volumes of 0.02 M Tris-acetate, pH = 6.5, for 12h each, at 4^{o}C, then transferred to sterile tubes and held at 4^{o}C until use.

All reagents and plastic-ware used in this study were free of endotoxin contamination as measured using the Limulus Amebocyte Lysate Assay (Associates of Cape Cod, Woods Hole, MA). The limit of detection of this assay is 0.025 □g/ml LPS.

^{7}Beryllium Bound in the Be-ferritin Adduct: The amount of ^{7}Be bound to the Be-ferritin adduct was calculated as described in the results from gamma counting using a Packard Cobra Auto-Gamma 5005 gamma counter (Canberra, Australia), of the ^{7}Be-ferritin reagent. In a subset of BAL cells from BeS (n = 3) and CBD (n = 1) subjects, we determined the sub-cellular distribution of 100 □M $^{7}BeCl_2$ and of the ^{7}Be-ferritin adduct reagent in whole cells, cell cytoplasmic and nuclear extracts prepared using the Active Motif Nuclear extraction kit (Carlsbad, CA).

Cell Cultivation: Bronchoalveolar lavage (BAL) was performed as previously described [E5]. Cells retrieved from the lung by BAL were cultured in complete medium (RPMI 1640 medium (Cambrex Bioproducts, Walkersville, MD) containing 10% iron supplemented calf serum (Hyclone, Logan, UT). 0.29 mg/ml L-glutamine, 100 U/ml penicillin G and 100 □g/ml streptomycin sulfate).

BAL macrophages and T cells were separated by the adherence method of Bost et al. [E6], with the following modification. Ten million BAL mixed cells were incubated in 10 ml of RPMI complete medium, in a 10 cm^2 petri dish (Falcon #3003), for 15 min at 37^oC in 5% CO_2. The non-adherent cells were removed using 3 X 10 ml washes of RPMI complete medium, the washes pooled, concentrated by centrifugation at 1200 rpm, the non-adherent cells suspended 1 ml of RPMI complete medium and held on ice. Ten ml of RPMI complete medium was added to the dish, and the adherent cells were removed using Costar (#3008) Cell Lifters, concentrated by centrifugation at 1200 rpm, suspended in 1 ml of RPMI complete medium and held on ice. Adherent cells prepared by this method consisted of 90 $\pm$ 2% non-specific esterase positive BAL macrophages and the non-adherent cells were 90 $\pm$ 2% non-specific esterase negative BAL lymphocytes. Cell numbers and viability were determined by hemocytometer counting of cells that excluded trypan blue dye, and was normally > 90% viable cells.

H36.12j cells (ATCC, CRL 2449) are clonally derived hybrid precursor macrophages derived from the fusion of drug selected P388D.1 (DBA/2, $H2^d$) macrophages with percoll gradient purified, proteose peptone elicited macrophages obtained from C57Bl/6N ($H2^b$) mice. H36.12j cells were cultivated in Dulbecco's Modified Eagle's medium (BioWhittiker, Walkersville, MD) supplemented with 10% heat inactivated calf serum, 0.29 mg/ml L-glutamine, 100 U/ml penicillin G, and 100 □g/ml streptomycin sulfate. Cell numbers and viability were determined as above.

Beryllium lymphocyte proliferation test (BeLPT): For clinical evaluation of Be sensitization, as presented in Table 1, the blood and BAL beryllium lymphocyte proliferation tests (BeLPT) were performed according to the clinical assay described by

Mroz et al. [E1]. Blood and BAL cells were adjusted to a concentration of 1 X 10^6 per ml of complete medium and 200 μl aliquots per well were then cultured in triplicate samples per treatment. Three plates were prepared in which the cells were unstimulated or exposed to 100 μM $BeSO_4$, 10 μM $BeSO_4$, 100 μM $Al_2(SO_4)_3$, or PHA (1 μg/ml). The treated plates were then incubated at 37°C in an humidified atmosphere containing 5% CO_2, and on days 4, 5 and 6 the cultures were pulse labeled with the DNA specific precursor tritiated thymidine deoxyriboside (^{3}HTdR; [methyl-^{3}H]-Thymidine, specific activity = 5.0 Ci/mmole; Amersham Biosciences, Piscataway, NJ) for 4 hr at 37°C in an atmosphere containing 5% CO_2. ^{3}H-DNA was harvested onto glass fiber filters using a Tomtec 96 well plate harvester, and the glass fiber filters were counted in a Packard TopCount NXT liquid scintillation counter (Packard Inst. Co., Meriden, CT). Thymidine uptake for the unstimulated controls on days 4, 5 and 6 are normally in the range of 150 cpm to 500 cpm. For the clinical evaluation of blood and BAL T cell proliferation in response to $BeSO_4$ stimulation shown in Table 1, we report the mean (± SEM) peak stimulation index (SI) for thymidine uptake as the ratio of the test sample counts per minute (cpm) to the cpm in the unstimulated (medium alone) control [E1].

In a separate experiment (presented in Figure 1), CBD (n = 8) BAL cell concentration was adjusted to 1 X 10^6/ml of complete culture medium and 200 μl aliquots were then cultured in triplicate per treatment. The cells were unstimulated (none), or treated with 100 μM $BeSO_4$, or the metal-salt control 100 μM $Al_2(SO_4)_3$. The prepared Be-ferritin adduct and the ferritin protein control reagents were diluted 1:20 in triplicate wells/treatment that contained test cells in 200 μl of complete medium. A 1:20 dilution of the dialysis control reagent, equal to the volume of the Be-ferritin adduct and protein

control reagents, was also transferred into a separate set of triplicate wells. This set of controls tested for the efficient removal of the unbound 0.1 M $BeSO_4$ by the dialysis step that was used to prepare the Be-ferritin adduct reagent. Thus, if the dialysis step did not effectively remove 0.1 M $BeSO_4$ from the dialysis buffer, then we would expect that amounts of $BeSO_4$ (> 10 μM to 100 μM) sufficient to induce Be-specific T cell proliferation should be present in this reagent. Alternatively, if the dialysis step removed unbound $BeSO_4$ from the dialysis buffer at levels < 10 μM $BeSO_4$ or at levels less than the amount of Be bound to Be-ferritin adduct, then dialysis control reagent should not induce Be-specific T cell proliferation.

The BeLPT was performed on days 4, 5 and 6 of culture, as described above, and for this experiment the peak thymidine incorporation cpm for each set of untreated and treated triplicate cultures were expressed as the mean (± SEM) cpm.

Apoptosis: H36.12j cells, BAL mixed cells, isolated BAL macrophage and isolated BAL lymphocyte concentrations were adjusted to 2.5 X 10^5/ml of complete medium, and 200 μl aliquots transferred into triplicate wells per treatment condition of 96 well round bottom culture dishes (No. 3799, Corning, NY). Cells were incubated at 37°C in an humidified atmosphere of 5% CO_2 under the following conditions: unstimulated (none), or exposed to 100 μM $BeSO_4$, a 1:20 dilution of the dialysis control reagent, a 1: 20 dilution of the ferritin protein reagent, 100 μM $Al_2(SO_4)_3$, a 1:20 dilution of the Be-ferritin adduct reagent. After 24 hr of exposure, cells were harvested by agitation, cytocentrifuge slides prepared and stained with a Protocol Hema 3 differential staining kit (Fisher Scientific, Denver, CO). Counts of 500 cells per treatment were used to determine the percent (mean ± SEM%) of cells with nuclear fragmentation. Confirmatory TUNEL

assays were performed using Dead End Assay kits (Promega Life Sciences, Madison, WI) according to the manufacturer's instructions. Using the cytocentrifuge slides, we determined the phagocytic index [PI = % of macrophages with apoptotic bodies X (number of apoptotic bodies/number of macrophages with apoptotic bodies); [E7] for unstimulated H36.12j cells and cells exposed to 100 μM $BeSO_4$, a 1:20 dilution of the ferritin protein control or a 1:20 dilution of the Be-ferritin adduct reagent. Confirmatory TUNEL staining was performed as above.

Intracellular staining for activated caspase was performed using Carboxyfluorscein caspase-3, -8 and -9 kits (BioCarta, Carlsbad, CA) with the following modifications. After intracellular caspase staining, cells were harvested by gentle agitation, pooled and concentrated by centrifugation. Pellets were suspended in 1 ml of ice cold sterile PBS, pH = 7.4, and concentrated by centrifugation. Pellets were then suspended in 1 ml of ice cold FACS-Block; 1X PBS, 10% heat inactivated fetal calf serum (Sigma), 1 mg/ml human gamma globulins (Sigma), and concentrated by centrifugation. The cell pellets were suspended in 100 μl of FACS-Block and 150 μl of FACS-Fix; 1X PBS + 1% paraformaldehyde was slowly added to fix the suspended cells, followed by an additional 100 μl of FACS-Block. Fixed cells were held overnight at 4°C in the dark until analysis.

For surface marker staining, 1 X 10^5 cells per marker, or per isotype control, were washed 3X by centrifugation at 1200 rpm in 1 ml of PBS, followed by 10 min incubation on ice in 1 ml of FACS-Block. The cells were concentrated by centrifugation, the pellet suspended in 100 μl of FACS-Block containing a 1:300 dilution of the labeled surface marker antibody or isotype control, and incubated in the dark for 15 min at 4°C. Labeling was stopped by the addition of 150 μl of ice cold FACS-Fix added slowly down the side

of the tube, and then brought to a final volume of 500 µl with ice cold FACS-Block. The fixed, labeled cells were held overnight at 4°C in the dark until analysis.

Intracellular caspase and surface marker analyses were performed using a Becton Dickinson FACSCalibur flow cytometer. Photomicroscopy was performed using a Nikon E600 microscope equipped with a Cool Snap digital imaging system and ImageQuant analysis software.

Statistics: A repeated measures ANOVA was used to determine the effect of treatments while adjusting for the variability of subjects. After the data were checked for significant treatment differences, individual contrasts were calculated to compare treatment means of interest. Normalizing transformations were made in cases where the data were non-Gaussian. When data transformations were unsuccessful, we used suitable nonparametric tests (nonparametric repeated measures ANOVA).

References

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Figure E1. The percent (mean% ± SEM) of CBD BAL cells (n = 8) with TUNEL positive nuclei (black) or with fragmented nuclei (open) after exposure to 100 μM $BeSO_4$. * $p < 0.05$ versus the corresponding unstimulated control, repeated measures ANOVA.

Figure E2. (a) The percent (mean% ± SEM) of CBD BAL (n = 6), mixed cells (black), adherent macrophages (open), and CBD BAL non-adherent lymphocytes (stripe) with fragmented nuclei 24h after exposure to the Be-ferritin adduct reagent that contained 270 picomoles of Be adducted to 50 μg of ferritin. * $p < 0.05$, for CBD BAL mixed cells and adherent CBD BAL macrophages versus their corresponding ferritin reagent control treated cells, nonparametric repeated measures ANOVA. (b) A photomicrograph showing the cytoplasmic blebbing (arrow 1) and nuclear fragmentation (arrow 2) of adherent CBD BAL macrophages (original 400X) and (c) the intact nuclei of non-adherent CBD BAL lymphocytes (original 400X), 24h after exposure to the Be-ferritin adduct reagent that contained 270 picomoles of Be adducted to 50 μg of ferritin.

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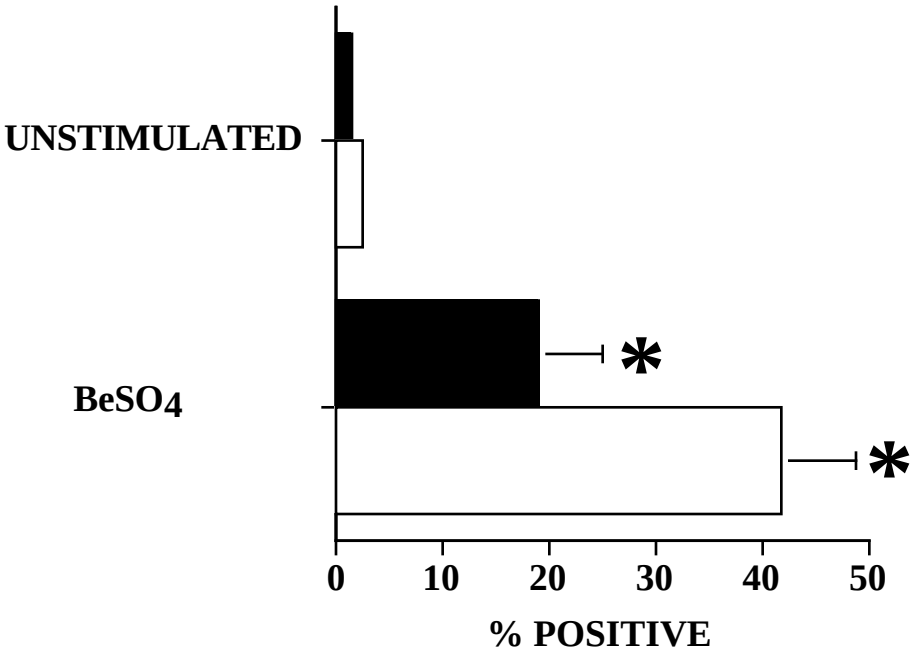

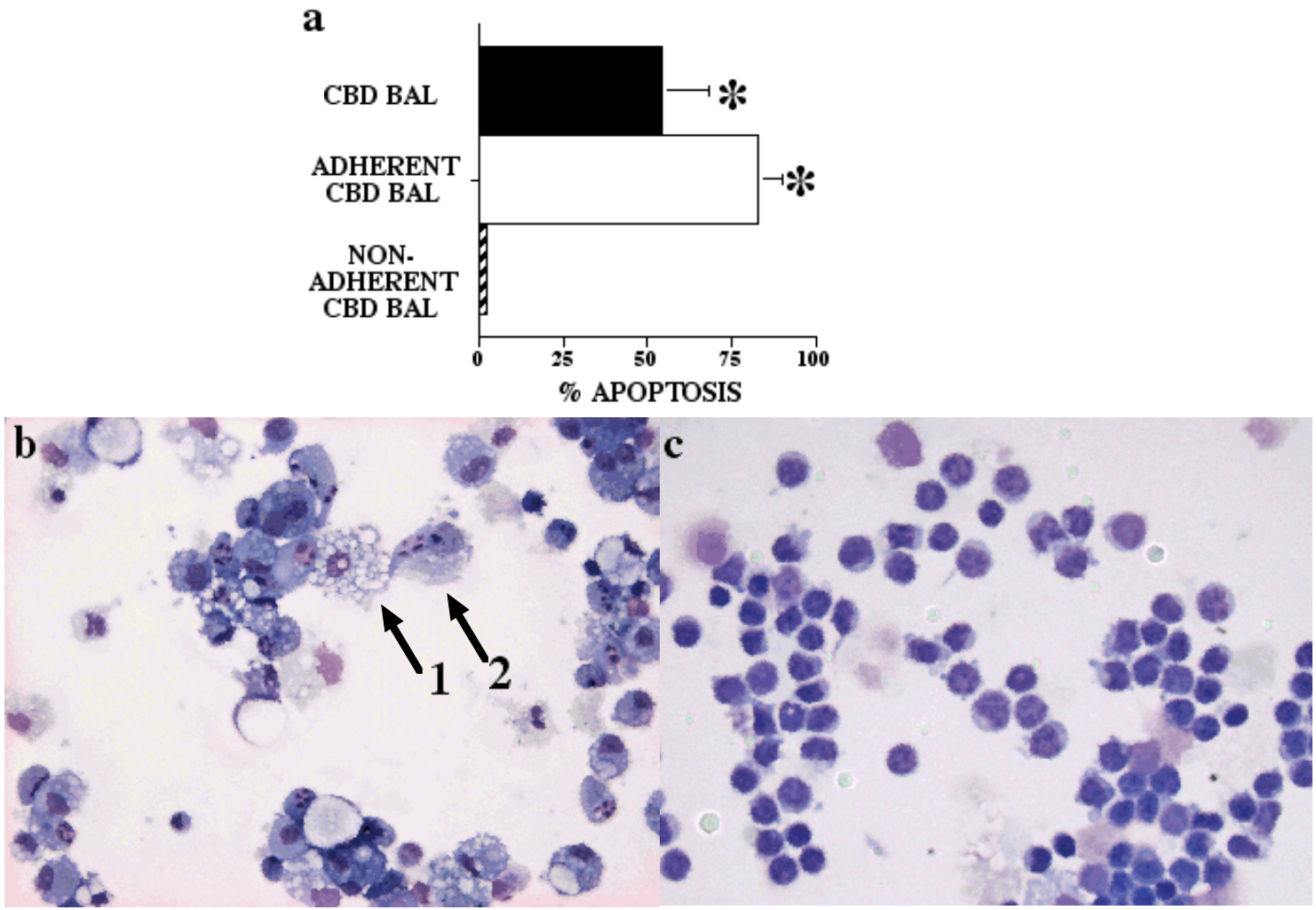
a
CBD BAL
ADHERENT CBD BAL
NON-ADHERENT CBD BAL
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*
0
25
50
75
100
% APOPTOSIS
b
1
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